ENZYMATIC DIGESTION, AN AID IN PRIMARY ISOLATION OF BACTERIA FROM DISEASED TISSUES

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An important function of any diagnostic laboratory is the isolation of pathogenic microorganisms from tissue specimens and their identification. A review of our experience over the past few years indicated that our standard methods for primary isolation of pathogens sometimes failed to demonstrate the presence of such organisms, particularly where case histories strongly indicated bacterial infection.

It was postulated that our routine methods may not be adequate to release bacteria from tissues when such organisms may be surrounded by normal or pathological cellular or tissue barriers.

It has been known for many years that the intercellular matrix of tissues can be digested with controlled amounts of proteolytic and hydrolytic enzymes (Monné, 1946; Day, 1947; Holtfreter, 1948; Essner *et al.*, 1954). Living microorganisms contained within these tissues will resist digestion, whereas dead cells are rapidly digested (Northrop *et al.*, 1948). Our objective was to determine whether diseased tissue specimens, when treated with certain proteolytic enzymes, would yield pathogenic microorganisms which otherwise might not be recovered by routine methods.

MATERIALS AND METHODS

Tissue specimens. Specimens were received from clinically diseased animals, in insulated containers, refrigerated during shipment either with dry ice or ice.

Enzymes. Crystalline trypsin and experimental enzyme X-108 (Cyanamid) were used in this study. Both were received as dried powders in vials.

X-108 is a powerful proteinase isolated from fermentation beer of *Condiobolus brefeldianus*. In the dry form, X-108 is stable at room temperature. In solution, 100,000 Azocoll units of X-108 per ml will lose about 50 per cent of their potency in 7 days at 4 C. A similar solution showed little loss of activity when held in the frozen state for 7 days.

The temperature range for proteolytic activity is 0 to 50 C; the optimum temperature, however, is about 37 C. The rate of proteolysis is lower at the lower temperature, whereas the enzyme exhibits instability at the higher temperatures. Optimum pH for its activity on casein is pH 8.0 to 8.5, although activity is good from pH 6.0 to 9.0.

The activity of a proteolytic enzyme may be determined by measuring the rate of digestion of its specific substrate. The Azocoll and casein assay methods were used to compare X-108 with trypsin. Using the Azocoll procedure (Bidwell, 1950), 1 μg nitrogen of X-108 gave approximately 700 to 800 Azocoll units of activity, whereas 1 μg nitrogen of crystalline trypsin gave approximately 350 Azocoll units at the optimum pH at 37 C (table 1). With the casein method (Spies and Chambers, 1951), X-108 brought about an increase of 40 per cent amino nitrogen during 40 hr incubation, whereas trypsin, at 320,000 units of activity per g of sodium caseinate, showed an increase of only 10 per cent amino nitrogen after 40 hr incubation.

These observations seemed to justify the use of X-108 and trypsin in these studies. Preliminary studies showed that pepsin quantitatively possessed considerably less activity and so was not included in this work.

Procedures. (1) Routine method:—Aseptically cut surfaces of small pieces of various organs are impressed on both horse blood agar and deoxycholate-citrate-lactose-sucrose agar plates. The smear is streaked across the plate with a sterile wire loop, and cultures are incubated. In addition, 0.5 ml of a 10 per cent suspension of pooled organs in peptone water is inoculated

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TABLE 1

Comparison of proteolytic activity of enzyme X-108 and trypsin

Enzyme Used	Azocoll Activity per μg Nitrogen of Enzyme	pH Optimum (Casein)	Mol Wt*	Half-life Stability at Optimal pH†	Optimum Temp	Isoelectric Point
X-108	400–800	8.5	30,000 ±	24 hr ±	37 C	pH 10.2
Crystalline trypsin	350 ±	8.0	36,000 ±	1 hr ±	37 C	pH 7.5

* Determined by Dr. R. Brown, Viral and Rickettsial Section, American Cyanamid Company.
† In absence of substrate.

intraperitoneally into 20 to 25 g Swiss mice which are held for 10 days. Heart blood from mice which die within the observation period in turn is impressed and streaked on culture plates.

Gram-negative organisms from either blood agar or deoxycholate-citrate-lactose-sucrose plates are transferred to triple sugar iron agar slants for detection of acid, gas, or hydrogen sulfide production. Species identification of both gram-positive and gram-negative bacteria is made from reactions in differential carbohydrate media following subculture from either plates or slants. This procedure covers aerobic cultivation.

For anaerobic cultivation, specimens are grown in either liquid thioglycolate medium or on blood agar plates, incubated under increased CO_2 tension. Species identification is made in the same manner as for the aerobes.

(2) Enzyme method:—Portions of spleen, kidney, lymph node, lung, and liver totaling 2 g from each case were minced with sterile scissors, and pooled.

Two g of pooled tissue were added to each concentration of enzymes: 1,000,000; 500,000; 250,000 units. The pH of the resultant tissue-enzyme mixture was 7.2. Mixtures were incubated for 1½ hr at 37 C. From each concentration of digested material, 0.2 ml was transferred by sterile pipette to blood agar and deoxycholate-citrate-lactose-sucrose plates, and liquid thioglycolate medium. Blood agar plates were incubated aerobically as well as anaerobically.

Simultaneously, 0.5 ml of each concentration was inoculated intraperitoneally into each of two 20 to 25 g mice for pathogenicity determination. As a control, tissues from the same animals, to which enzymes were not added, were streaked on plates, and a 10 per cent pooled suspension was inoculated into mice, according to the routine method.

Preliminary studies. Before enzymes could be considered as a tool in primary isolation, certain basic questions were explored: (a) Were the enzymes free of bacteria? (b) Did enzymes have any adverse effect on bacteria in pure culture or in tissues?

(1) Sterility of enzymes:—Serial dilution from 10^{-1} to 10^{-6} of both enzymes were plated out on blood agar and incubated under aerobic and anaerobic conditions for sterility test. Both enzymes were found to be free of microbial contaminants.

(2) Effect of enzymes on pure cultures of bacteria:—Enzyme X-108 and crystalline trypsin were reconstituted in 10 ml of physiological saline at concentrations of 1,000,000, 500,000, and 250,000 Azocoll units, respectively. The pH was 7.2. Dilutions of both enzymes were made and placed in 0.5-ml amounts in both media, corresponding to dilutions of 10^{-1} through 10^{-4}, containing *Salmonella choleraesuis* var. *kunzendorf*, *Pasteurella multocida*, and *Corynebacterium pyogenes*, respectively. Following incubation at 37 C for 48 hr, the organisms were subcultured to blood and deoxycholate-citrate-lactose-sucrose agar plates which were, in turn, also incubated at 37 C for 48 hr. Abundant growth was recovered from all tubes, indicating that neither enzyme, under these conditions, showed a bactericidal effect. Subcultures of the organisms demonstrated that there was no bacteriostatic activity.

(3) Effect of enzymes on bacteria in tissues:—Two-g portions of pooled organs, from which *Salmonella typhimurium* had previously been recovered, were treated with 1,000,000, 500,000, and 250,000 units of X-108 and trypsin, in 10 ml of saline, respectively, and incubated at 37 C for 1½ hr. In addition, as a control, 2 g of pooled organs in 10 ml of saline were incubated simultaneously in the same manner. Following

TABLE 2

Quantitative recovery of Salmonella typhimurium from tissues

Enzyme Used	Units	Total Organisms Recovered
X-108	1,000,000	750,000
X-108	500,000	346,000
X-108	250,000	28,000
Trypsin	1,000,000	286,000
Trypsin	500,000	73,000
Trypsin	250,000	11,000
None (Saline)	—	3,540

TABLE 3

Quantitative recovery of organisms from sheep tissues

Enzyme Used	Units	Total Organisms Recovered
X-108	1,000,000	56,000
X-108	500,000	None
X-108	250,000	None
Trypsin	1,000,000	None
Trypsin	500,000	None
Trypsin	250,000	None
None (Saline)	—	None

the incubation period, each pool of tissue was subcultured to blood agar plates in 0.2-ml amounts, after diluting the suspension to 10^{-5}. Time counts were made after the plates were incubated at 37 C for 24 hr. Using *S. typhimurium* as the type organism, results were obtained as shown in table 2.

This technique was utilized to ascertain whether the recovery of the bacteria was actually due to enzymatic digestion or bacterial multiplication under incubation at 37 C.

To further establish this point, the same procedure was utilized with pooled organs from a sheep in which bacteria were not isolated under routine procedures, although post-mortem examination and clinical data indicated a septicemia. The problem of bacterial isolation was further complicated by the fact that the animal in question had been treated with tetracycline previous to its death. The results obtained are shown in table 3.

Similar selected control cases gave essentially the same results. These results indicated that X-108 and trypsin, in some cases, enhanced bacterial recovery.

RESULTS OF THE APPLICATION OF THE ENZYME METHOD

Table 4 summarizes the isolation of organisms from five specimens by the use of enzyme X-108 and crystalline trypsin, and by the routine method (control).

Case no. D4136—swine. The clinical history indicated that a salmonellosis was probably present. However, when tissues were handled according to the routine method, no pathogens were recovered. The use of X-108 resulted in isolation of *P. multocida* (23×10^3), (11.6×10^3) and *S. typhimurium* (43×10^4), (21×10^4), at the two higher concentrations, respectively. Trypsin-treated tissue, on the other hand, presented the same results as the untreated control tissues. Identical results were obtained when the pH of the diluent was adjusted to 8.0 to 8.5.

All mice died 30 min after inoculation with X-108 treated tissue, whereas control mice survived the 10-day observation period. With trypsin treated tissue inoculum, at the 1,000,000 unit concentration, mice died within 30 min; at 500,000 units, within 24 hr; and at 250,000 units, survived the 10-day observation.

Case no. D4244—swine. Pathogens were not recovered from the untreated control, nor from the trypsinized samples. When X-108 was added, *S. choleraesuis* var. *kunzendorf* (31×10^4), (9.6×10^3), was only recovered with the two higher concentrations. The mouse inoculation results with X-108 and trypsin were the same as in case no. D4136. Inoculated mice withstood trypsinized inoculum to a greater degree than X-108 inoculum, as in the first case.

Case no. D4404—deer. The case history and autopsy finding suggested pasteurellosis. Pathogens were not recovered from the tissues by the routine method, nor from trypsinized specimens. However, *P. multocida* (27×10^3), (8.2×10^3), was recovered from all concentrations of X-108. Inoculated mice followed the same death pattern as in previous cases.

Case no. D4214—swine. The case history and the death of the inoculated mice within 48 hr indicated the presence of a possible bacteremia, but no etiological agent was recovered from either swine tissues or mice by the routine method. Addition of X-108, at the 1,000,000 and 500,000 unit levels, resulted in the isolation of *P. multocida* (16.9×10^3), (3.1×10^3). Trypsin, at 1,000,000 units, also resulted in the recovery of the organ-

TABLE 4

Isolation of organisms from five specimens

Specimen No.	Enzyme Used	*Staphylococcus albus*	*Pseudomonas* spp.*	*Pasteurella multocida*	*Salmonella typhimurium*	*Salmonella choleraesuis* var. *kunzendorf*	Coliforms*	*Cryptococcus* spp.*	*Corynebacterium pyogenes* (β-Hemolytic)	*Bacillus* spp.*
1	X-108:									
	1,000,000 units	+	+	+	+					
	500,000 units	+	+	+	+					
	250,000 units	+	+	−	+					
	Trypsin, all concentrations	+	+	−	−					
	Control	+	+	−	−					
2	X-108:									
	1,000,000 units	+				+	+			
	500,000 units	+				+	+			
	250,000 units	+				−	+			
	Trypsin, all concentrations	+				−	+			
	Control	+				−	+			
3	X-108, all concentrations			+			+			+
	Trypsin, all concentrations			−			+			+
	Control			−			+			+
4	X-108:									
	1,000,000 units	+		+			+	+		
	500,000 units	+		+			+	+		
	250,000 units	+		−			+	+		
	Trypsin:									
	1,000,000 units	+		+			+	−		
	500,000 and 250,000 units	+		−			+	−		
	Control	+		−			+	−		
5	X-108:									
	1,000,000 units					+			+	
	500,000 units					+			+	
	250,000 units					−			−	
	Trypsin, all concentrations					−			−	
	Control					−			−	

* Nonpathogenic.

ism (12.5×10^3), whereas the two lower levels of trypsin did not.

Case no. D4149—encapsulated abscess from cattle. This specimen was included to demonstrate the feasibility of enzymatic digestion of abscess material as an aid in isolation of microorganisms. Although results with the routine method and the addition of trypsin were negative, X-108 yielded *S. choleraesuis* var. *kunzendorf* (29.4×10^4), (17.7×10^4), and *C. pyogenes* (25.8×10^3), (8×10^3), at the two higher concentrations.

Other cases. Five additional case specimens were subjected to similar treatment. Combining these cases with those above, we summarize as follows. Pathogens were recovered from tissues, untreated with enzyme, from 2 out of 10 cases. With X-108, pathogens were recovered from 8 of the 10 cases, usually at the two highest levels, at pH 7.2 to 8.5.

Toxicity of enzymes to mice. X-108-treated suspensions (pH 7.2 to 8.5), when inoculated intraperitoneally in 0.5-ml amounts at all three levels into 20- to 25-g Swiss mice resulted in the death of the animals within 30 min postinocula-

tion. With crystalline trypsin suspension (pH 7.2 to 8.5) at the 1,000,000 concentration, death occurred within 30 min; at 500,000 units, within 24 hr; whereas those receiving 250,000 units survived. In the cases reported, all mice survived inoculation with tissue suspensions without enzymes. These enzyme-tissue results are in agreement with previous mouse tests where enzymes alone at the same dosage levels were employed, and indicate that the mortality is directly associated with the enzymes. It may be further observed that in this series of cases, mouse inoculation was far inferior to enzyme digestion in demonstrating the presence of pathogens.

DISCUSSION

This study shows that some bacterial pathogens are missed when purely physical or mechanical procedures are utilized for isolation purposes. Apparently normal or pathologic cellular barriers prevent isolation by these methods. Although the impression of tissue on plate media may be considered a sampling of tissue fluids, it has been our experience that tissue prepared by Ten Broeck grinding has not significantly improved isolation success over the impression method.

It is probable that the principal action of enzymes in facilitating bacterial isolation is due to a breaking down of cellular barriers. After the death of an animal, the entrance of enzymes through the cell membrane is facilitated, probably because of increased permeability resulting from chemical and physical alterations in the protein linkages (Osterhout, 1922). This action apparently aids in releasing organisms which may be present within the intracellular as well as the intercellular matrix. Other investigators have reported that while surrounding tissues undergo proteolysis, living organisms in these areas remain impervious to enzymatic activity. Our results confirm these reports. Serial dilutions of pathogens recovered from enzyme-treated tissues were inoculated intraperitoneally into mice; death occurred in the usual time for each organism.

Our studies suggest another possible beneficial effect of enzymatic digestion. With continued enzymatic action, amino acids become available to the bacteria present with apparent enhancement of their growth.

P. multocida was readily isolated from enzyme X-108-digested tissue held at 4 C for 2 months. Normally, under these conditions, without enzymes, isolation was successful only with difficulty.

The type of enzyme selected is important. It is interesting to note that when tissues were treated with X-108, pathogens were usually recovered. However, in every instance but one, these results were not duplicated with trypsinized tissues. The following hypothesis is offered as a possible explanation of this phenomenon.

The proteolytic activity of X-108 extends over a longer period than trypsin and this is believed to be a significant factor in the results obtained. Furthermore, it is reasonable to assume that various portions of the protein linkages are attacked in a more efficient manner with reference to penetrability of the cell membrane than is possible with trypsin. It is well known that crystalline trypsin undergoes rapid self-digestion when in solution. On the other hand, X-108 apparently does not suffer from such rapid self-digestion. In addition, the amount of trypsin necessary to duplicate the activity of a given amount of X-108 would necessitate a much larger quantity of the enzyme and even then, the resulting digestion would probably not continue sufficiently long to release deeply imbedded bacteria.

Although incubation of the enzyme X-108-treated tissue suspension at room temperature for several hours is often satisfactory, incubation at 37 C for 1½ hr appears optimal. If enzyme-treated tissues are not transferred to plates immediately, the suspension should be stored at 4 C. This will result in further disintegration of the remaining enzyme. The inoculum may be transferred, even after several months of storage, to various media with apparently little damage to the living bacteria.

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SUMMARY

Successful application of enzyme digestion as an aid in primary isolation of bacteria from

tissues is principally dependent upon the selection of a suitable enzyme used at adequate concentrations. Enzyme X-108 at concentrations of 1,000,000 and 500,000 Azocoll units per 2 g of tissue, with incubation at 37 C for 1½ hr, markedly increased isolation success over the routine method. Enzyme X-108 at a concentration of 250,000 Azocoll units per g of tissue did not increase isolation. Trypsin used at equivalent concentrations and conditions was not significantly superior to the routine method. The enzyme method described is considered a practical laboratory procedure for the isolation of both aerobic and anaerobic bacteria.

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